Journal of Chromatography A, 1217 (2010) 4612–4622

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of anthelmintic drug residues in milk using ultra high performance liquid chromatography–tandem mass spectrometry with rapid polarity switching

Michelle Whelan[a,b], Brian Kinsella[a,b], Ambrose Furey[b], Mary Moloney[a], Helen Cantwell[a], Steven J. Lehotay[c], Martin Danaher[a,*]

[a] *Food Safety Department, Teagasc, Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland*
[b] *Team Elucidate, Department of Chemistry, Cork Institute of Technology, Bishopstown, Cork, Ireland*
[c] *US Department of Agriculture, Agriculture Research Service, Eastern Regional Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA*

ARTICLE INFO

Article history:
Received 12 January 2010
Received in revised form 16 April 2010
Accepted 3 May 2010
Available online 6 May 2010

Keywords:
Anthelmintics
UHPLC–MS/MS
Polarity switching
QuEChERS
Milk

ABSTRACT

A new UHPLC–MS/MS (ultra high performance liquid chromatography coupled to tandem mass spectrometry) method was developed and validated to detect 38 anthelmintic drug residues, consisting of benzimidazoles, avermectins and flukicides. A modified QuEChERS-type extraction method was developed with an added concentration step to detect most of the analytes at <1 $\mu g\,kg^{-1}$ levels in milk. Anthelmintic residues were extracted into acetonitrile using magnesium sulphate and sodium chloride to induce liquid–liquid partitioning followed by dispersive solid phase extraction for cleanup. The extract was concentrated into dimethyl sulphoxide, which was used as a keeper to ensure analytes remain in solution. Using rapid polarity switching in electrospray ionisation, a single injection was capable of detecting both positively and negatively charged ions in a 13 min run time. The method was validated at two levels: the unapproved use level and at the maximum residue level (MRL) according to Commission Decision (CD) 2002/657/EC criteria. The decision limit (CCα) of the method was in the range of 0.14–1.9 and 11–123 $\mu g\,kg^{-1}$ for drugs validated at unapproved and MRL levels, respectively. The performance of the method was successfully verified for benzimidazoles and levamisole by participating in a proficiency study.

1. Introduction

Several anti-parasitic drugs are licensed for treating helminth (parasitic worm) infections in food-producing animals. These can be broadly classified as nematicides, flukicides and endectocides. Only a limited number of products are licensed for treatment of animals during the lactating period and have a maximum residue limit (MRL) listed under European Commission Regulation 37/2010. These drugs are listed in Table 1. The widespread availability of cheaper generic veterinary medicinal products and the development of drug resistance [1,2] to the limited number of licensed products have increased the potential for off-label applications [3]. In addition, there is concern that residues from products applied during dry cow period can persist in milk post-calving. It has been reported that some of these substances have undesirable toxic effects at high doses in laboratory animals, namely, teratogenic or goitrogenic effects [4–6]. However, levels detected in food are generally well below toxicity thresholds and pose no risk to the consumers, but there are concerns about the presence of residues in milk.

Several analytical methods have been developed to detect anthelmintic residues in milk using a range of technologies such as ELISA [7], SPR-biosensor [8–10] and HPLC [11–13]. Recently, several groups have developed LC–MS/MS methods for detecting residues in milk [14–18]. These methods typically require large volumes of solvent [19], purification of extracts on solid-phase extraction (SPE) cartridges and/or test for a specific class of anthelmintic residues [20]. Many of these methods are limited in the number of samples that they can process by the 24-sample-footprint of conventional SPE manifold blocks. Some groups have improved sample throughput through introducing more costly automated SPE platforms [21,22]. However, major drawbacks including scope of methods and the volumes of waste solvent generated have not been addressed.

Recently, our group (Kinsella et al.) developed a simple extraction procedure for isolating 38 anthelmintic drugs residues from milk using the QuEChERS ("quick, easy, cheap, effective, rugged and safe") method widely used in pesticide residue analysis [23]. The

* Corresponding author. Tel.: +353 1 8059500; fax: +353 1 8059550.
E-mail address: martin.danaher@teagasc.ie (M. Danaher).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.05.007

Table 1
MRLs for anthelmintic drug residues in milk as listed under European Commission Regulation 37/2010.

Veterinary drug	Marker residue(s)	MRL (μg kg^{-1})
Albendazole, albendazole-sulphoxide, netobimin	Sum of albendazole-sulphoxide, albendazole-sulphone and albendazole-sulphone amine expressed as albendazole	100
Fenbendazole, oxfendazole, febantel	Sum of fenbendazole, fenbendazole-sulphoxide and fenbendazole-sulphone expressed as fenbendazole-sulphone	10
Thiabendazole	Sum of thiabendazole and 5-hydroxythiabendazole	100
Morantel	Morantel	50
Oxyclozanide	Oxyclozanide	10
Eprinomectin	Eprinomectin B_{1a}	20
Moxidectin	Moxidectin	40

QuEChERS approach has many advantages over existing extraction procedures due to its quick easy extractions where contaminants, water, proteins and fats are removed and analytes are extracted into MeCN in one step while maintaining high recoveries. MeCN was chosen as the extraction solvent as it separates from water in the presence salt (NaCl) and also extracts very little fat. Another advantage of QuEChERS is dispersive solid phase extraction (DSPE) as it is quicker and easier than traditional solid phase extraction (SPE) which is time consuming and requires careful attention. DSPE reduces extraction time, labour, costs, solvent, waste, glassware and minimal training is required compared to SPE. This QuEChERS method (Kinsella et al.) was transferred to our laboratory and was intended for routine use. However the lower sensitivity for macrocyclic lactones in milk (limit of quantitation of 5 μg kg^{-1}) compared with an existing in-house HPLC fluorescence method (limit of quantitation of 2 μg kg^{-1} for avermectins) had limited the application of this method as we required a more sensitive method. In addition, limitations of the method developed by Kinsella et al. included the requirement for separate injections for negatively and positively ionised substances, and the longer run time that is typical of LC–MS/MS analysis.

In recent years, several methods have been developed using UHPLC offering improved separation of analytes in short run times [24–27]. These improvements in peak capacity have been achieved in such separations through the introduction of sub-2 μm particles and higher linear velocities delivered by high pressure LC pumps [28]. UHPLC applications in the area of multi-residue analysis have been facilitated by prior developments in fast scanning mass spectrometers, which allow a suitable number of data points (12–15) to be simultaneously acquired for several peaks that are a few seconds wide [29]. Furthermore, several instruments now include more stable detector power sources, which allow the possibility of fast polarity switching with minimal delays as low as 20 ms to enable detection of both positively and negatively charged analytes in a single injection. However, few multi-residue methods have been published in peer-review literature, which take advantage of this approach [30].

In this paper, we describe the development of a new method to detect anthelmintic residues in milk using UHPLC–MS/MS with fast polarity switching. The method allows for the detection of both positively and negatively ionised compounds in a 13 min run time. The method now allows the detection of anthelmintic drug residues to <1 μg kg^{-1} through careful optimisation of mobile phase additives and introduction of a concentration step using dimethyl sulphoxide (DMSO) as a keeper to overcome protein binding and ensure analytes remain in solution during evaporation. The suitability of the method has been evaluated through application to milk samples from treated animals.

2. Experimental

2.1. Reagents and materials

Ultra-pure water (18.2 MOhm) was generated in-house using a Millipore (Cork, Ireland) water purification system. HPLC-grade methanol (MeOH) and acetonitrile (MeCN), 99.5% deuterated MeOH, and ammonium formate (puriss pro analysis) were sourced from Sigma–Aldrich (Dublin, Ireland). Analar grades (analytical grades) of DMSO, isopropyl-alcohol (IPA), and glacial acetic acid (HOAc) were obtained from BDH Chemicals Ltd. (Poole, UK). Pre-weighed mixtures of 4 g anhydrous (anh.) magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) in 50 mL centrifuge tubes, and 1.5 g anh. $MgSO_4$ and 0.5 g of C_{18} bulk sorbent in 50 mL centrifuge tubes were obtained from UCT, Inc. (Bristol, PA; USA). Organic milk was purchased in supermarkets and tested for residues prior to method development.

2.2. Standards, internal standards and stock solutions

Abamectin (ABA), albendazole (ABZ), bithionol (BITH), clorsulon (CLOR), closantel (CLOS), coumaphos (COUM), doramectin (DORA), emamectin (EMA), fenbendazole (FBZ), haloxon (HAL), ivermectin (IVER), levamisole (LEVA), morantel (MOR), niclozamide (NICL), nitroxynil (NITR), oxfendazole (OFZ), oxyclozanide (OXY), rafoxanide (RAF), and thiabendazole (TBZ) were purchased from Sigma–Aldrich. Albendazole-2-amino-sulphone (ABZ-NH_2-SO_2), albendazole-sulphone (ABZ-SO_2), albendazole-sulphoxide (ABZ-SO), amino-oxibendazole (OXI-NH_2), 5-hydroxy-thiabendazole (5-OH-TBZ), fenbendazole-sulphone (FBZ-SO_2), triclabendazole (TCB), triclabendazole-sulphone (TCB-SO_2) and triclabendazole-sulphoxide (TCB-SO) were purchased from Witega Laboratories Berlin-Aldershof GmbH (Berlin, Germany). Coumaphos-oxon (COUM-O) was purchased from Greyhound Chromatography and Allied Chemicals (Merseyside, UK). Cambendazole (CAM) and oxibendazole (OXI) were purchased from QMX Laboratories (Essex, UK). Amino-flubendazole (FLU-NH_2), amino-mebendazole (MBZ-NH_2), hydroxy-flubendazole (FLU-OH), hydroxy-mebendazole (MBZ-OH), flubendazole (FLU) and mebendazole (MBZ) were donated by Janssen Animal Health (Beerse, Belgium). Eprinomectin (EPR) was donated by Merial Animal Health (Lyon, France). Moxidectin (MOXI) was donated by Fort Dodge Animal Health (Princeton, NJ, USA).

Deuterated forms of several of the analytes were used as internal standards. Albendazole-D3 (ABZ-D3), albendazole-sulphone-D3 (ABZ-SO_2-D3), albendazole-sulphoxide-D3 (ABZ-SO-D3), fenbendazole-D3 (FBZ-D3), fenbendazole-sulphone-D3 (FBZ-SO_2-D3), fenbendazole-sulphoxide-D3 (FBZ-SO-D3), levamisole-D5 (LEVA-D5), mebendazole-D3 (MBZ-D3), thiabendazole-D3 (TBZ-D3) and triclabendazole-D3 (TCB-D3) were purchased from

Witega Laboratories. Albendazole-2-amino-sulphone-D2 (ABZ-NH_2-SO_2-D2) was sourced from QUCHEM (Belfast, UK). Other internal standards included selamectin (SELA), which was donated by Pfizer Animal Health (New York, NY; USA), salicylanilide (SALI), 4-Nitro-3-(trifluoromethyl)phenol (TFM), and ioxynil (IOX), which were purchased from Sigma–Aldrich. Amino-triclabendazole (TCB-NH_2) was sourced from Witega.

Primary stock standard solutions were prepared at concentrations of 4000 $\mu g\,mL^{-1}$ from the certified standard materials; ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, EPR, CLOS, OXY, NITR, CLOR, BITH and MOR. The remaining standards were prepared at concentrations of 2000 $\mu g\,mL^{-1}$. All internal standards were prepared at concentration of 1000 $\mu g\,mL^{-1}$. Avermectins were prepared in MeCN, flukicides, CAM, LEVA and TCB metabolites were prepared in MeOH and the remaining benzimidazoles prepared in DMSO.

Intermediate working standard mix solutions were prepared at a concentration of 100 $\mu g\,mL^{-1}$ for OXY, CLOR, BITH and MOR and 50 $\mu g\,mL^{-1}$ in MeOH for the remaining analytes. A working internal standard mix was prepared at the following concentrations: 20 $\mu g\,mL^{-1}$ for SELA and TCB-NH_2, 4 $\mu g\,mL^{-1}$ for LEVA-D5, TBZ-D3 and IOX, 2 $\mu g\,mL^{-1}$ for the remaining analytes in MeOH-D (we used deuterated MeOH in case of deuterium exchange in solution). Primary, intermediate and working standard solutions are stable for at least six months when stored at −20 °C.

Extracted matrix calibrants were prepared by fortifying negative milk samples prior to extraction with working standard mixes, prepared at the following concentrations (in $\mu g\,mL^{-1}$): 5 (Std 6), 2.5 (Std 5), 1 (Std 4), 0.5 (Std 3), 0.25 (Std 2), and 0.1 (Std 1) (OXY, CLOR, BITH and MOR were twice as concentrated in each solution). For low level validations, matrix-matched calibration curves were prepared by fortifying matrix blanks before extraction with 100 μL of the standards to give working standard curves in the sample equivalent range of 1–50 $\mu g\,kg^{-1}$ (or 2–100 for OXY, CLOR, BITH and MOR). For validations at the MRLs, an additional two matrix-matched points were added to the curve by fortifying with 200 and 400 μL of Std 6 to give a working standard curve in the range of 1–200 $\mu g\,kg^{-1}$ (or 2–400 for OXY, CLOR, BITH and MOR). These extracted matrix-matched calibration curves were used to obtain the validation data. An additional four blank matrix samples (recovery controls) were fortified after extraction, two with Std 2 (50 μL) and two with Std 5 (50 μL) to monitor for loss of analytes during extraction. All samples and controls were spiked with internal standard prior to extraction.

2.3. Apparatus

A glass dispenser (Dispensette® III, Brand) was used for aliquoting MeCN extraction solvent, a Mistral 3000i centrifuge, a multi-vortexer, a Caliper Life Sciences (Runcorn, UK) Turbovap LV evaporator, and a Transsonic 780LH ultrasonic bath were used for the extraction.

Separations were performed using a Waters (Milford, MA, USA) Acquity UPLC system comprising of a stainless steel HSS T3 analytical column (100 mm × 2.1 mm, particle size 1.8 μm) equipped with an in-line filter unit containing a 0.2 μm stainless steel replacement filter maintained at a temperature of 60 °C and the pump was operated at a flow rate of 0.6 $mL\,min^{-1}$. A binary gradient system was used to separate analytes comprising of mobile phase A, 0.01% HOAc in water:MeCN (90:10, v/v) and mobile phase B, 5 mM ammonium formate in MeOH:MeCN (75:25, v/v). The gradient profile was as follows: (1) 0–0.5 min, 100% A, (2) 5 min, 50% A, (3) 7 min, 10% A, (4) 8.5 min, 10% A, (5) 8.51 min, 0% A, (6) 9.5 min, 0% A, (7) 9.51 min, 100% A, (8) 13 min 100% A. Injection volume was 5 μL.

The veterinary drug residues and their metabolites were quantified using a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) interface. The UHPLC–MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ Software (both from Waters).

Parent ion masses were calculated from the elementary compositions. The optimum collision energies were obtained during tuning and they were inputted into the MS settings. The SRM windows were time-sectored, and dwell time, inter-scan delay and inter-channel delays were set to get maximum response from the instrument. These conditions can be seen in Table 2.

2.4. Sample preparation

Milk samples (10 ± 0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard and left to sit for 15 min. The samples were extracted into MeCN (12 mL), $MgSO_4$ (4 g) and NaCl (1 g). Samples were shaken immediately and centrifuged for 12 min at 3500 RPM (2842 g). A dispersive-SPE cleanup step was performed by pouring the supernatant into a centrifuge tube (50 mL) containing $MgSO_4$ (1.5 g) and C_{18} (0.5 g). The samples were vortexed for 30 s and centrifuged for 10 min at 2500 RPM (1449 g). The supernatant (6 mL) was added to an evaporation tube containing DMSO (0.25 mL) and vortexed for 1 min. MeCN was evaporated under nitrogen at 50 °C using the Turbovap apparatus to 0.25 mL. Extracts were filtered through 0.2 μm PTFE 13 mm syringe filters (Whatman Rezist®) and injected onto the UHPLC–MS/MS system.

2.5. Validation procedure

The method was validated according to European Legislation 2002/657/EC [31]. The following performance studies were carried out: specificity, within-laboratory repeatability (WLr) and reproducibility (WLR), linearity, recovery, decision limit (CCα) and detection limit (CCβ). A low level validation study was carried out using samples fortified at 1, 1.5 and 2 times the second lowest calibration level (LCL), which was 4 $\mu g\,kg^{-1}$ for OXY, CLOR, BITH and MOR and 2 $\mu g\,kg^{-1}$ for the remaining analytes. A second MRL level validation study was carried out at 0.5, 1 and 1.5 times the MRL.

3. Results and discussion

3.1. Method development

MS conditions were initially optimised by infusing standards at concentration of 1 $\mu g\,mL^{-1}$ and tuning the cone and collision energies using mobile phase A containing 12.5 mM ammonium formate in water:MeCN:MeOH (90:5:5, v/v) and mobile phase B containing 12.5 mM ammonium formate in MeOH:MeCN (50:50, v/v). In previous work, ammonium formate was found to be a suitable mobile phase additive for this group of analytes [23]. Acidic modifiers were also evaluated but were found to be unsuitable for avermectins due to the formation of sodium adducts. As a result, a more detailed experiment was designed to investigate the effect of different mobile phase additives on sensitivity.

A central composite design experiment was carried out to optimise mobile phase additives (Table 3). The following factors were selected for optimisation: (a) HOAc, 0–1% in mobile phase A; (b) ammonium formate, 0–12.5 mM in mobile phase B; and (c) % MeOH in MeCN, 50–100% in mobile phase B. Mobile phase was prepared as described in Table 3 and extracted samples were injected using the various mobile phase conditions. The results were analysed using central composite design. As a result, of the negative effect of acidic additives on the ionisation of avermectins during tuning, HOAc and ammonium formate were added to mobile phase A and B separately.

Table 2
UPLC–MS/MS conditions.

Analyte	t_R (min)	Transition (m/z)	Dwell time (s)	Cone (V)	CE (V)	MRM window	ESI polarity	IS
LEVA-D5	1.58	210.10 → 183.08	300	40	20	1	+	IS
LEVA	1.59	204.93 → 122.89	300	35	27	1	+	LEVA-D5
		204.93 → 177.94	300	35	14	1	+	
5-OH-TBZ	1.59	217.87 → 146.87	8	45	32	1	+	ABZ-NH_2-SO_2-D2
		217.85 → 190.85	8	45	24	1	+	
ABZ-NH_2-SO_2-D2	1.62	242.00 → 133.00	5	40	30	1	+	IS
ABZ-NH_2-SO_2	1.63	240.08 → 133.15	5	40	27	1	+	ABZ-NH_2-SO_2-D2
		240.08 → 198.10	5	40	20	1	+	
MOR	2.55 and 2.98	220.95 → 110.90	50	30	25	2	+	TBZ-D4
		220.95 → 122.93	50	30	33	2	+	
OXI-NH_2-D7	2.93	199.25 → 109.05	5	40	30	2	+	IS
TBZ-D4	3.08	205.99 → 179.00	80	47	24	2	+	IS
NITR	3.13	288.90 → 126.86	5	37	23	3	–	IOX
		288.90 → 161.95	5	37	22	3	–	
TBZ	3.13	201.90 → 130.85	5	45	32	2	+	TBZ-D4
		201.90 → 174.8	5	45	24	2	+	
ABZ-SO-D3	3.26	285.25 → 243.02	5	41	13	4	+	IS
ABZ-SO	3.28	282.24 → 159.06	5	27	35	4	+	ABZ-SO-D3
		282.24 → 240.10	5	27	15	4	+	
CLOR	3.29	377.70 → 341.95	5	25	12	3	–	SALI
		379.80 → 343.95	5	23	12	3	–	
MBZ-NH_2	3.34	238.10 → 105.09	5	50	24	4	+	TCB-NH_2 Pos
		238.10 → 133.05	5	50	34	4	+	
ABZ-SO_2-D3	3.60	301.00 → 158.95	5	40	38	4	+	IS
ABZ-SO_2	3.62	298.10 → 159.08	5	42	35	4	+	ABZ-SO_2-D3
		298.10 → 266.20	5	42	20	4	+	
FLU-NH_2	3.66	256.06 → 95.10	35	45	34	4	+	TCB-NH_2 Pos
		256.06 → 123.05	35	45	26	4	+	
FBZ-SO-D5	4.07	321.04 → 158.95	23	30	32	4	+	IS
OFZ	4.10	316.10 → 159.05	5	35	30	4	+	FBZ-SO-D5
		316.10 → 191.09	5	35	24	4	+	
MBZ-OH-D3	4.25	301.15 → 160.05	5	36	32	4	+	IS
MBZ-OH	4.27	298.25 → 160.05	5	38	33	4	+	MBZ-OH-D3
		298.25 → 266.15	5	38	22	4	+	
FBZ-SO_2-D5	4.42	337.06 → 305.00	5	45	23	5	+	IS
FBZ-SO_2	4.45	331.90 → 158.90	5	35	36	5	+	FBZ-SO_2-D5
		331.90 → 300.00	5	35	21	5	+	
FLU-	4.54	316.20 → 125.10	8	40	33	5	+	MBZ-OH-D3
OH		316.20 → 160.05	8	40	35	5	+	
IOX	4.55	369.65 → 126.80	35	35	33	6	–	IS
CAM	4.73	302.96 → 216.85	5	35	26	5	+	FBZ-D5
		302.96 → 260.95	5	35	18	5	+	
OXI-D7	4.99	257.15 → 177.05	5	32	28	5	+	IS
OXI	5.04	249.90 → 175.90	7	35	26	5	+	OXI-D7
		249.90 → 218.00	7	35	18	5	+	
TFM	5.16	205.95 → 159.95	35	37	24	6	–	IS
MBZ-D3	5.18	299.15 → 105.05	5	39	33	5	+	IS
MBZ	5.19	296.14 → 105.05	5	35	32	5	+	MBZ-D3
		296.14 → 264.10	5	35	18	5	+	
FLU-D3	5.41	317.15 → 123.00	5	40	36	5	+	IS
FLU	5.43	313.80 → 123.00	5	40	35	5	+	FLU-D3
		313.80 → 282.00	5	40	24	5	+	
SALI	5.65	212.05 → 92.00	30	35	28	6	–	IS
ABZ-D3	5.85	269.12 → 233.85	5	35	19	5	+	IS
ABZ	5.86	266.07 → 191.03	5	33	32	5	+	ABZ-D3
		266.07 → 234.00	5	33	13	5	+	
COUM-O	6.04	347.01 → 210.99	5	30	29	7	+	TCB-NH_2 Pos
		347.01 → 291.02	5	30	22	7	+	

Table 2 (*Continued*)

Analyte	t_R (min)	Transition (m/z)	Dwell time (s)	Cone (V)	CE (V)	MRM window	ESI polarity	IS
HAL	6.18	414.90 → 211.00	10	40	35	7	+	TCB-NH_2 Pos
		414.90 → 272.95	10	40	32	7	+	
FBZ-D5	6.23	305.01 → 273.01	5	28	15	7	+	IS
FBZ	6.28	300.01 → 159.01	5	35	24	7	+	FBZ-D5
		300.01 → 268.01	5	35	23	7	+	
TCB-NH_2 Pos	6.36	328.00 → 166.95	5	48	27	7	+	IS
TCB-NH_2 Neg	6.36	325.87 → 180.90	5	45	26	8	–	IS
TCB-SO_2	6.62	389.00 → 244.16	5	55	28	8	–	TCB-NH_2 Neg
		389.00 → 309.94	5	55	27	8	–	
OXY	6.63	397.80 → 175.75	5	32	26	8	–	SALI
		397.80 → 201.80	5	32	20	8	–	
TCB-SO	6.67	375.03 → 181.00	5	35	40	8	–	TCB-NH_2 Neg
		375.03 → 212.86	5	35	30	8	–	
NICL	6.88	324.95 → 170.91	5	33	26	8	–	SALI
		324.95 → 288.89	5	33	17	8	–	
COUM	6.89	363.02 → 227.05	5	35	25	7	+	TCB-NH_2 Pos
		363.02 → 307.05	5	35	16	7	+	
TCB-D3	6.97	361.90 → 343.90	5	43	25	7	–	IS
TCB	6.98	359.04 → 274.07	5	45	36	7	+	TCB-D3
		359.04 → 343.97	5	45	27	7	+	
BITH	7.08	352.75 → 160.70	5	32	23	7	–	TFM
		352.75 → 191.70	5	32	26	7	–	
CLOS	7.11	660.85 → 126.90	5	45	43	8	–	SALI
		660.85 → 315.10	5	45	35	8	–	
RAF	7.30	623.79 → 344.83	10	58	33	8	–	SALI
		623.79 → 126.90	10	58	36	8	–	
EMA	7.49	886.54 → 126.05	5	50	38	9	+	SELA
		886.54 → 158.01	5	50	37	9	+	
EPR	7.71	915.15 → 144.06	10	19	41	9	+	SELA
		915.15 → 298.15	10	19	18	9	+	
ABA	7.84	890.50 → 305.15	25	14	25	9	+	SELA
		890.50 → 567.10	25	14	13	9	+	
MOXI	8.02	640.25 → 498.30	8	15	12	9	+	SELA
		640.25 → 528.40	8	15	8	9	+	
DORA	8.05	916.60 → 331.30	18	17	22	9	+	SELA
		916.60 → 593.35	18	17	12	9	+	
SELA	8.30	770.40 → 333.30	20	40	22	9	+	IS
IVER	8.38	892.25 → 307.35	32	15	20	9	+	SELA
		892.25 → 569.45	32	15	13	9	+	

Table 3
Factorial design experiments for optimisation of mobile phase additives.

Experiment	HOAc (%)	Formate (mM)	MeOH (%)
1	0.5	6.25	75
2	0.5	6.25	55
3	0.9	6.25	75
4	0.1	6.25	75
5	0.5	11.25	75
6	0.5	6.25	75
7	0.5	1.25	75
8	0.5	6.25	95
9	0.5	6.25	75
10	0.5	6.25	75
11	1.0	0	50
12	1.0	12.5	100
13	0	0	50
14	0.5	6.25	75
15	1.0	12.5	50
16	0	0	100
17	0	12.5	100
18	0	12.5	50
19	0.5	6.25	75
20	1.0	0	100

The results of the central composite design experiment are shown in Fig. 1 for the least intense analytes using the chosen mobile phase conditions. The y value shows the factor of improvement in concentration using the additives described above. In general, it was found that lower concentrations of mobile phase additives resulted in more efficient ionisation of analytes, and indeed, the majority of analytes were ionised best in the absence of mobile phase additives. For example, clorsulon increases by a factor of 20 with no additives compared to a factor of 6.2 with the current mobile phase additives. However, when deciding on the final mobile phase additives a compromise had to be made as mobile phase additives were required to improve chromatography and reduce peak tailing for some analytes.

The signal intensity for a number of analytes (ABA, CLOR, DORA, EPR, IVER, NITR, OXY and TCB-SO_2) was significantly weaker and required careful optimisation to improve sensitivity. Mobile phase conditions were first optimised for ABA, DORA, EPR and IVER. Optimisation experiments estimated that best overall response for avermectins could be achieved using a mobile phase B containing 5 mM ammonium formate and a solvent composition of

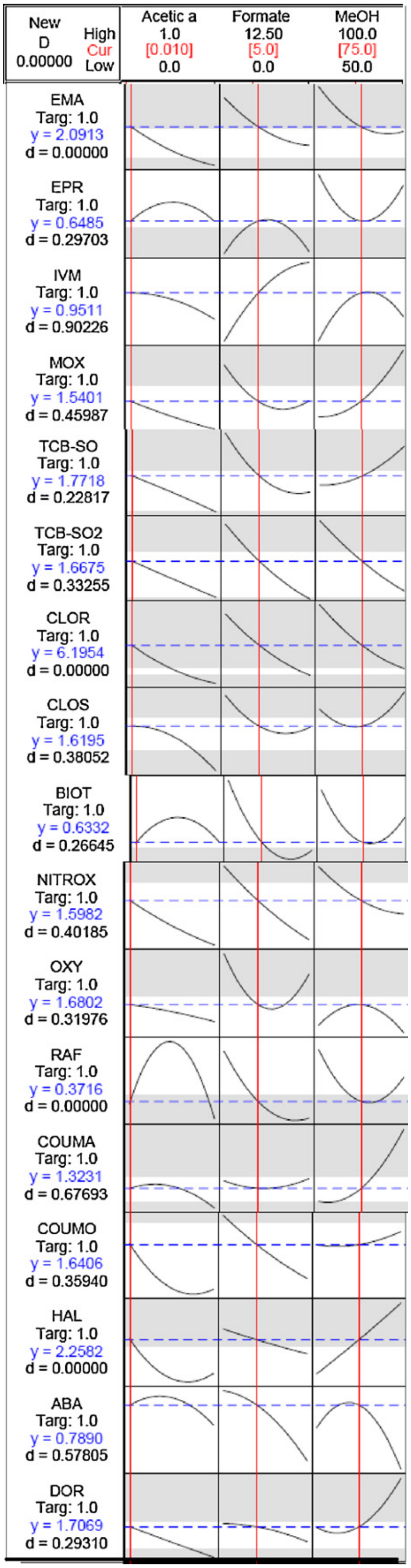

Fig. 1. Response surface model: effect of mobile phase additives (HOAc, ammonium formate and MeOH in MeCN) on MS response. The model was developed from the results of the experiments outlined in Table 3.

MeOH:MeCN (75:25, v/v). In agreement with earlier work [23], it was found that concentrations of HOAc in the eluent should be kept to a minimum to ensure a good response for avermectins. This was achieved using a mobile phase A of 0.01% HOAc in H_2O:MeCN (90:10, v/v). In the case of some poorly responsive compounds, namely, CLOR, NITR, OXY and TCB-SO_2 sensitivity was improved by as much as 20-, 3.3-, 2.5- and 2.25-fold, respectively.

The set-up of the SRM windows for this method was complex for two reasons: the 76 ion transitions for the analytes, and the inclusion of polarity switching to accommodate ESI positive and negative modes in the same injection. In addition, SRM sectoring was further complicated by the additional 23 transitions needed for internal standards. Internal standards were included to improve accuracy and repeatability through negating the effects of ion suppression (potential false negatives) and enhancement effects (potential false positives). A further benefit of internal standards is that they provided continuous monitoring of the performance of the chromatograph and mass spectrometer during analysis. [32,33]

A total of 12–15 data points were typically obtained across a peak to attain reproducible integration and, thus achieve highly repeatable quantitative analysis. SRM conditions were established through effective set-up of dwell times, inter-scan delay and inter-channel delay. The inter-scan delay was set to 5 ms between successive SRM windows of the same polarity and 20 ms when switching polarity. The number of data points across peaks was typically controlled by varying dwell times. Dwell times ranged between 200 ms (LEVA) and 5 ms for more complex scanning segments of the chromatogram (Table 2). This would not have been possible on older instruments, which are incapable of rapid polarity switching and detecting both positively and negatively charged ions in a single injection due to switching times of 200–700 ms compared to 20 ms. Although setting up a polarity switching method is more difficult than existing stand-alone positive or negative ionisation methods, this approach has many advantages as it eliminated the use of multiple injections and longer run times which results in faster turnaround times. Analytes are eluted in under 9 min with run times of 13 min (Fig. 2). This new polarity switching approach also reduces solvent usage. The sensitivity and resolution of the method is also increased significantly in comparison to HPLC methods.

The sensitivity of the method was further improved through introduction of a 20-fold concentration of extracts. This was achieved by extracting milk samples with 12 mL MeCN and scaling up the dispersive-SPE step to 10 mL of extract with 500 mg of C_{18} sorbent. The supernatant (6 mL) was transferred to test-tubes containing 0.25 mL of DMSO and concentrated to a constant volume of 0.25 mL. The key to this step was the addition of DMSO to the sample extract before concentration, which acts as a keeper to ensure analytes remain in solution and to reduce the potential for protein binding. A further advantage of DMSO extract over MeCN extract is that the volume injected could be increased (from 2 to 5 µL) without band broadening effects.

3.2. Method validation

The method was validated according to European Commission Decision 2002/657/EC criteria. The specificity of the method was investigated through monitoring for interferences in UHPLC–MS/MS traces from analytes or internal standards. Transitions for ABZ-SO_2 (m/z 298.1 → 266.2) and MBZ-OH (m/z 298.25 → 266.15) were prone to isobaric interference but were sufficiently separated in UHPLC–MS/MS traces (3.57 min vs. 4.14 min, respectively). The absence of cross-talk interference was verified by injecting analytes and internal standards separately. Morantel also contained two peaks at the following retention times (Rt); 2.55 and 2.98 min (Table 2). These peaks were identified as the trans

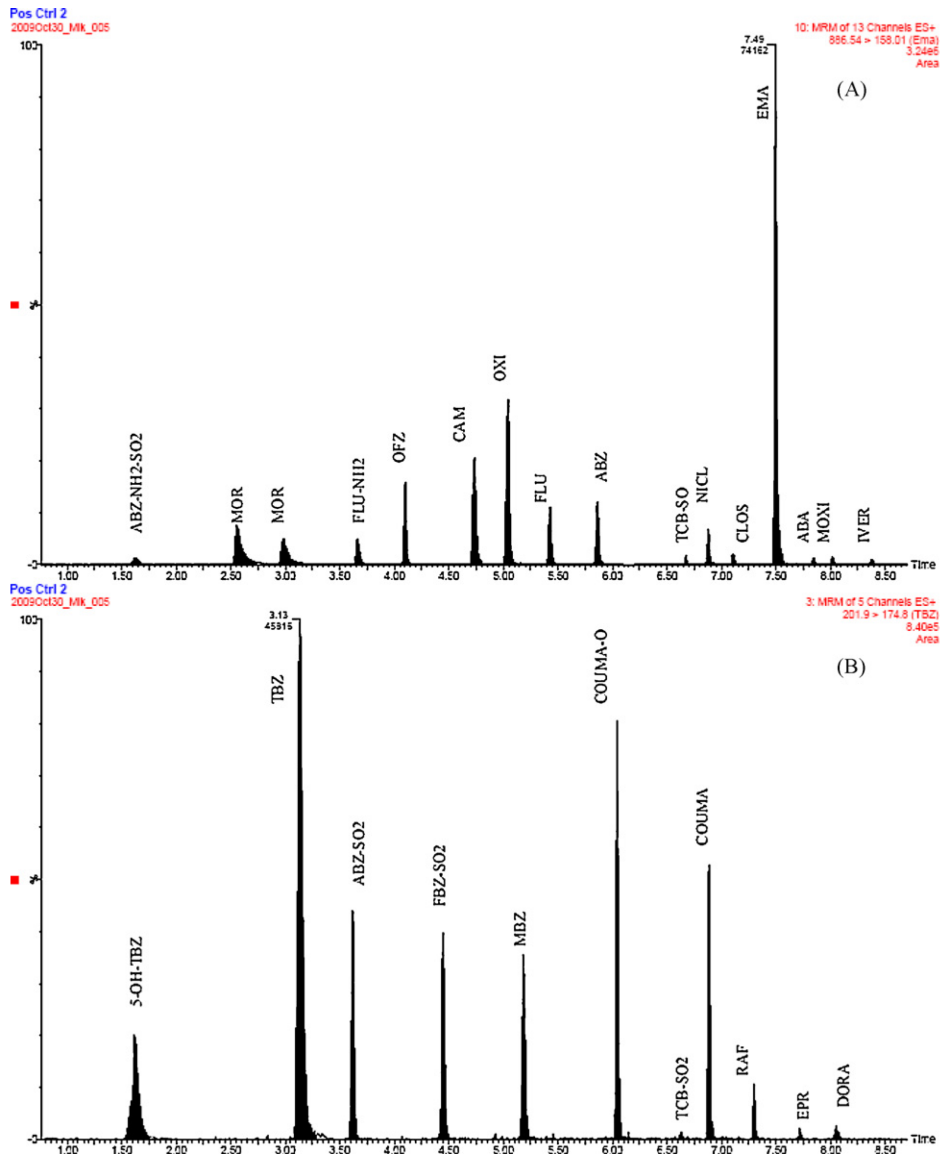

Fig. 2. Overlay of analytes (A–C) at concentration of 2 μg kg^{-1} (OXY, CLOR, BITH and MOR were 4 μg kg^{-1}) and internal standards (D–E).

and cis isomer, respectively. It was observed that the second peak (Rt 2.98 min) was larger than the first peak (Rt 2.55 min) for the matrix calibrants fortified prior to extraction, and the first peak was larger than the second peak for the recovery controls spiked at the end of the extraction. This is due to degradation of morantel through photo-isomerisation in the presence of ultraviolet (UV) light from the biologically active trans isomer (first peak) to the biologically inactive cis isomer (second peak) [34,35]. As a result, both peaks were summed to calculate the concentration of Morantel in the samples. The selectivity of the method was initially evaluated through application to 20 different bovine milk samples received from different sources without observed interferences. The method has since been applied to more than 500 milk test samples.

The linearity of the method was evaluated over the range 0–200 μg kg^{-1} during validation studies. The linearity of curves measured as R^2 were typically greater than 0.99. The calibration curves for some negatively ionised analytes were linear over a shorter range. TCB-SO_2 was linear only in the range of 1–10 μg kg^{-1}and either quadratic or bracketed calibration would be used for calculations at higher concentrations in this case. However, the majority of negative ionised substances including TCB-SO_2 were not approved for use in lactating animals or had MRL values that fell within the linear part of the calibration curves.

A number of experiments were carried out to investigate the reason for the reduced linearity for TCB-SO_2 including: (a) different mobile phase additives, (b) flow rate (through flow reduction and splitting), and (c) source parameters (temperature, capillary and cone voltages). None of these experiments improved the linear dynamic range for the analyte. Results indicated likely causes of poor linearity were (a) inefficient ionisation of analytes and/or (b) saturation of the detector. The authors propose that this linearity can be addressed through improvements in UHPLC–MS/MS instrumentation, namely, improvements in the electrospray probe, source construction or detector. An alternative approach is to investigate the use of different mobile phase additives.

3.2.1. Low level validation precision

The WLr study was carried out by a single analyst, the method was repeated on three separate days for each of the validations. The WLR study was carried out by three different analysts on three separate days for each of the validations (total of six validation runs). In each study (WLr and WLR), negative milk samples were fortified

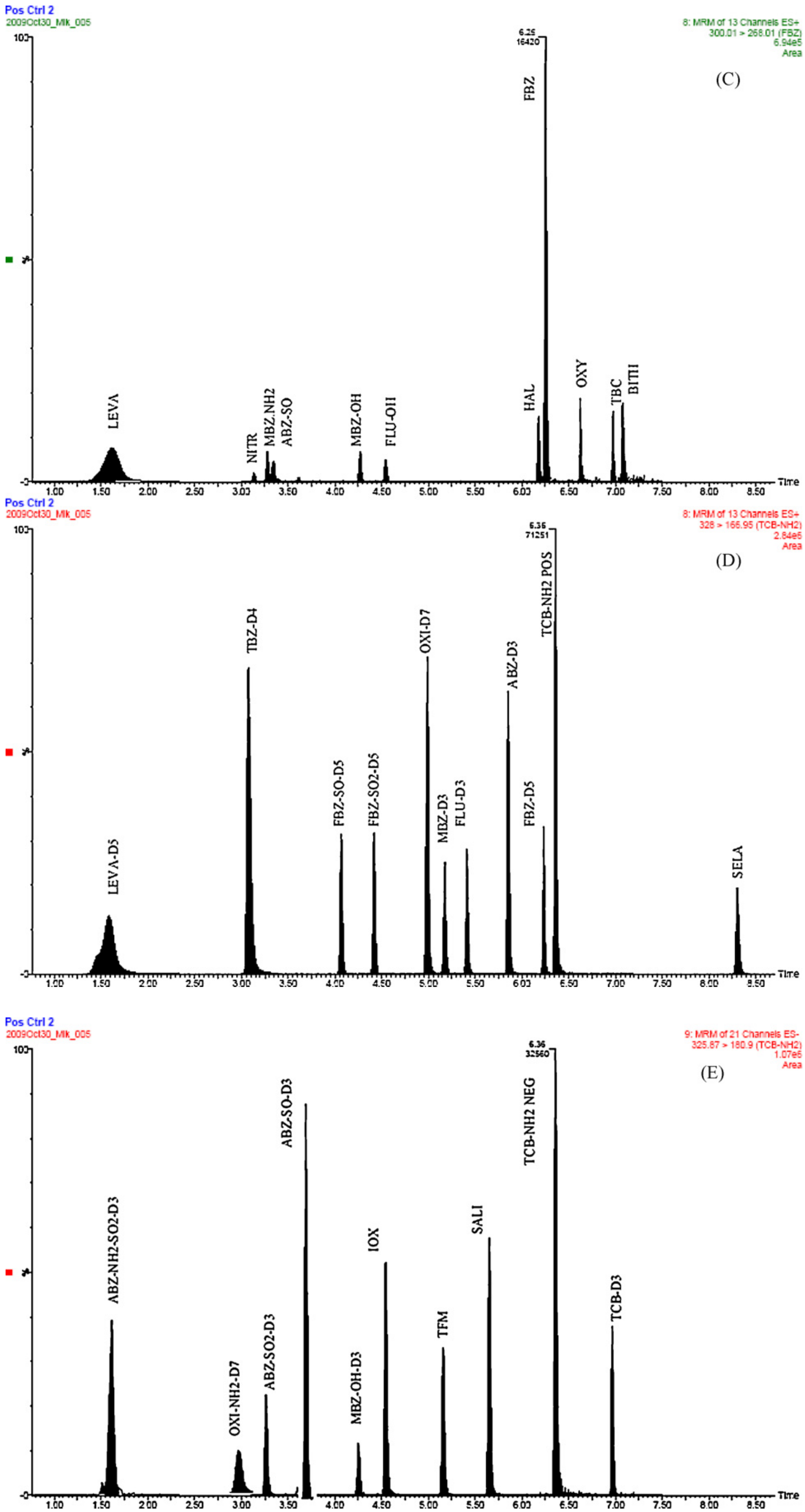

Fig. 2. (*Continued*).

Table 4
Banned level validation study results for reproducibility (WLR) and repeatability (WLr).

Analyte	Validation levels ($n = 6$) (μg kg^{-1})			Recovery (%)						CV (%)					
	1× 2nd LCL	1.5× 2nd LCL	2× 2nd LCL	1× 2nd LCL		1.5× 2nd LCL		2× 2nd LCL		1× 2nd LCL		1.5× 2nd LCL		2× 2nd LCL	
				WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr
ABZ	2	3	4	99	105	98	103	98	104	3.1	12	3.6	6.6	4.7	8.6
ABZ-SO	2	3	4	103	109	99	104	104	106	9.0	13	14	11	11	9.6
ABZ-SO_2	2	3	4	96	106	99	104	98	106	7.1	10	6.1	7.9	6.3	6.9
ABZ-NH_2-SO_2	2	3	4	104	105	108	110	112	116	11	11	9.6	11	9.3	18
CAM	2	3	4	104	113	105	113	107	110	8.3	10	6.5	5.8	7.8	9.7
FBZ	2	3	4	99	110	100	106	100	105	4.1	14	4.6	11	3.3	11
OFZ	2	3	4	95	102	97	102	99	103	5.6	16	6.6	11	5.2	7.8
FBZ-SO_2	2	3	4	96	101	95	99	97	101	5.6	11	7.2	7.0	6.0	7.1
FLU	2	3	4	111	109	93	102	98	101	19	9.5	16	8.5	26	6.4
FLU-NH_2	2	3	4	106	104	107	103	106	105	12	11	15	10	9.7	11
FLU-OH	2	3	4	97	108	95	109	96	107	10	9.8	13	7.8	9.7	5.6
MBZ	2	3	4	98	106	98	104	97	105	3.7	11	4.9	8.0	4.9	7.9
MBZ-NH_2	2	3	4	103	108	107	104	104	101	18	20	18	14	13	12
MBZ-OH	2	3	4	96	107	98	108	98	103	6.1	13	4.8	9.8	7.4	10
OXI	2	3	4	128	114	104	105	107	107	24	16	25	9.8	42	7.6
TCB	2	3	4	96	106	97	104	96	103	5.2	10	5.5	6.5	4.8	8.2
TCB-SO	2	3	4	104	104	106	114	115	111	27	27	9.9	23	16	19
TCB-SO_2	2	3	4	136	146	139	146	129	121	20	34	13	27	14	18
TBZ	2	3	4	97	101	87	96	89	101	18	18	26	19	22	16
5-OH-TBZ	2	3	4	96	107	99	109	98	119	33	38	33	34	32	32
LEV	2	3	4	104	103	97	97	94	98	18	14	18	13	21	11
BITH	4	6	8	98	112	113	104	110	109	31	19	30	6.8	26	11
CLOR	4	6	8	109	114	131	117	143	120	22	33	18	28	28	23
CLOS	2	3	4	67	96	80	105	85	112	25	27	17	14	26	8.0
MOR	4	6	8	95	98	88	96	89	96	11	14	13	12	8.4	9.4
NICL	2	3	4	100	104	109	110	112	110	13	13	11	8.7	17	8.7
NITR	2	3	4	99	123	102	135	107	137	14	41	4.7	27	5.5	29
OXY	4	6	8	109	127	111	132	108	125	12	40	6.8	26	12	30
RAF	2	3	4	91	99	96	104	93	106	9.7	16	12	9.4	15	5.9
COUM	2	3	4	100	96	101	95	99	93	9.0	14	13	8.1	9.4	8.5
COUM-O	2	3	4	103	102	109	99	113	101	15	16	18	11	18	11
HAL	2	3	4	98	94	99	96	100	94	15	19	17	11	16	11
ABA	2	3	4	90	99	92	100	95	100	15	21	9.0	17	9.1	12
DORA	2	3	4	91	96	91	98	94	99	16	20	15	18	10	12
EMA	2	3	4	93	94	92	102	91	103	6.0	9.2	8.9	6.2	7.0	9.6
EPR	2	3	4	96	97	92	99	98	103	8.1	18	7.2	11	9.7	14
IVER	2	3	4	98	116	98	115	100	119	11	19	7.1	21	10	24
MOXI	2	3	4	88	91	93	98	92	98	29	32	17	19	17	17

at 1.0, 1.5 and 2.0 times the second lowest calibration level (2nd LCL: 2–4 μg kg^{-1}) ($n = 6$ at each level) as described in 2002/657/EC [31]. Mean recoveries were typically in the acceptable region of 70–110%. Recovery for FBZ, OXI, TCB-SO, TCB-SO_2, 5-OH-TBZ, BITH, CLOR, CLOS, NITR, OXY, and IVER was greater than 110% in the WLr study (Table 4). Recovery for ABZ-NH_2-SO_2, FLU, OXI, TCB-SO, TCB-SO_2, BITH, CLOR, NICL and COUMA-O were greater than 110% and CLOS was less than 70% for the WLR study (Table 4). According to the validation criteria, the precision of the method at 100 μg kg^{-1} should be ≤23% and should be as low as possible for levels <100 μg kg^{-1}. Higher CVs were observed for the WLr compared with the WLR results. It is proposed that this related to the low levels being measured in samples and the performance of the instrument at the time of analysis. The WLr and WLR precision was <23% for the majority of analytes. WLr was >23% for TCB-SO, TCB-SO_2, 5-OH-TBZ, CLOR, CLOS, NITR, OXY, and MOXI. WLR was >23% for FLU, OXI, TCB-SO, 5-OH-TBZ, BITH, CLOR, CLOS, and MOXI.

3.2.2. MRL validation precision

The method was also validated a second time at MRL levels for analytes containing an MRL. In the MRL validation, negative milk samples were fortified as described in Table 5 at 0.5×, 1× and 1.5× MRL ($n = 6$ at each level). Two studies were also carried out for WLr and WLR, which involved the completion of WLR validation studies by three different analysts, and WLr by one analyst, each on three separate days (total of six validation runs) at the MRL. Typical WLR and WLr mean recovery was in the acceptable region of 70–110%. In contrast, to the low level validation, CVs for the WLR and WLr MRL validation study were generally in close agreement. The recovery and precision for most substances in the WLR and WLr validation study were still in the acceptable range (Table 5). The exception was the recovery for OXY which was just below acceptable levels of 70% and OFZ was just above the acceptable level of 110% and WLR precision was >23% for OXY. For the WLr study the exception was the recovery for OXY, which was just below acceptable levels of 70% and its WLr precision was >23%.

3.2.3. Decision limit (CCα) and detection capability (CCβ).

The decision limit (CCα) is defined as the limit above which it can be concluded with an error of probability of α, that a sample contains the analyte. The detection capability (CCβ) is defined as the lowest concentration of analyte at which the method is able to detect and quantify contaminated samples with a statistical certainty of 1-β. [31]. CCα and CCβ values for unapproved substances were calculated using the intercept (value of the signal, y, where the concentration, x, is equal to zero) and the standard error of the intercept (SEI) for a set of data with six replicates at three levels (1×, 1.5× and 2× 2nd LCL). CCα is the concentration corresponding to the intercept + 2.33 × the SEI. CCβ is the concentration corresponding to the signal at CCα + 1.64 × the SEI. CCα and CCβ for MRL substances were calculated using the calibration procedure for marker residues according to ISO 11843 for a set of data with six replicates at three levels (0.5, 1.0 and 1.5 MRL). CCα is the concentration calculated from the response at the MRL + 1.64 × the WLR

Table 5
MRL level validation study results for reproducibility (WLR) and repeatability (WLr).

Analyte	Validation levels (n = 6) (μg kg^{-1})			Recovery (%)						CV (%)					
	0.5× MRL	1× MRL	1.5× MRL	0.5× MRL		1× MRL		1.5× MRL		0.5× MRL		1× MRL		1.5× MRL	
				WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr	WLR	WLr
ABZ	50	100	150	94	97	94	98	95	98	3.5	7.3	4.9	7.9	5.0	6.5
ABZ-SO	50	100	150	93	106	88	95	85	95	5.7	10	11	9.1	8.5	9.7
ABZ-SO_2	50	100	150	96	104	94	99	95	97	4.4	5.0	5.4	7.5	6.3	7.1
ABZ-NH_2-SO_2	50	100	150	95	99	102	100	105	102	6.5	4.4	4.2	6.1	11	7.8
FBZ	5	10	15	109	102	116	107	115	105	6.5	4.7	4.4	7.0	8.2	4.7
OFZ	5	10	15	107	98	115	103	114	105	10	4.7	9.8	6.6	7.1	5.1
FBZ-SO_2	5	10	15	104	102	109	107	109	107	4.3	4.8	5.7	10	5.4	4.1
TBZ	50	100	150	102	104	101	101	97	97	4.0	6.9	3.1	5.9	3.8	7.6
5-OH-TBZ	50	100	150	94	97	85	86	80	81	5.2	8.5	8.2	12.9	8.9	12.5
MOR	25	50	75	84	95	85	95	82	89	7.7	4.3	9.8	7.5	12	15
OXY	5	10	15	61	66	59	62	58	71	25	39	23	33	18	33
EPR	10	20	30	94	90	96	96	95	96	6.1	6.9	5.5	5.3	6.7	7.9
MOXI	20	40	60	101	97	101	100	101	97	5.7	9.9	9.1	11	7.9	11

standard deviation (SD). CCβ is the concentration calculated from the response at CCα + 1.64 × the WLR SD.

CCα and CCβ values for unapproved and MRL substances are listed in Table 6. CCα is the main factor for a quantitative confirmatory method because it is the level above which a test result is declared non-compliant. The CCα values ranged from 0.14 to 1.9 and 11 to 123 μg kg^{-1} for unapproved and MRL substances, respectively. CCβ values ranged from 0.27 to 3.2 and 12 and 146 μg kg^{-1} for unapproved and MRL substances, respectively.

Table 6
Summary of validation data.

Analyte	Calibration range (μg kg^{-1})	Linearity r^2	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
ABZ	1–200	0.997	107	117
ABZ-SO	1–200	0.993	123	146
ABZ-SO_2	1–200	0.997	105	116
ABZ-NH_2-SO_2	1–200	0.995	105	120
CAM	1–50	0.994	0.27	0.46
FBZ	1–200	0.996	11	12
OFZ	1–200	0.999	12	14
FBZ-SO_2	1–200	0.998	11	12
FLU	1–50	0.998	1.0	1.7
FLU-NH_2	1–50	0.995	0.44	0.75
FLU-OH	1–50	0.995	0.41	0.69
MBZ	1–50	0.998	0.18	0.31
MBZ-NH_2	1–50	0.993	0.58	1.0
MBZ-OH	1–50	0.995	0.24	0.41
OXI	1–50	0.990	1.6	2.8
TCB	1–50	0.997	0.19	0.33
TCB-SO	1–25	0.987	0.55	0.94
TCB-SO_2	1–10	0.984	0.60	1.0
TBZ	1–200	0.998	108.2	115.3
5-OH-TBZ	1–200	0.999	114.7	135.5
LEV	1–50	0.995	0.83	1.4
BITH	2–50	0.990	1.9	3.2
CLOR	2–100	0.986	1.4	2.4
CLOS	1–50	0.994	0.68	1.2
MOR	2–400	0.998	58	71
NICL	1–50	0.994	0.49	0.84
NITR	1–25	0.981	0.24	0.41
OXY	2–50	0.984	14	20
RAF	1–50	0.991	0.49	0.83
COUM	1–50	0.988	0.40	0.68
COUM-O	1–50	0.990	0.59	1.0
HAL	1–50	0.992	0.58	0.98
ABA	1–50	0.993	0.35	0.60
DORA	1–50	0.989	0.45	0.77
EMA	1–50	0.996	0.29	0.49
EPR	1–50	0.986	22	25
IVER	1–50	0.989	0.34	0.59
MOXI	1–100	0.991	45	52

3.2.4. Application of the method to incurred samples

The suitability and reproducibility of the method was evaluated by analysing incurred milk samples and the performance of the method was verified by participating in a proficiency study according to ISO 5725-2. The method was verified in a proficiency study organised by the Community Reference Laboratory. A total of 20 laboratories participated in this study. The study consisted of six lyophilised milk samples, one of which was negative. The remaining samples contained the following incurred residues, LEVA, ABZ, FBZ, TCB and their metabolites (unknown to the analysts). All six samples were identified correctly, no false positive or false negative results obtained. *Z*-score values obtained by our laboratory were in the range of ±1.

The method was also applied to incurred milk samples from an animal dosed subcutaneously with Closamectin^{TM} (0.2 mg IVER and 5 mg CLOS kg^{-1} bodyweight). The method was shown to successfully detect residues in collected samples. IVER residues were detectable above CCα in milk samples at levels of 122, 80 and 21 μg kg^{-1} for 1, 2, and 4 days post-treatment. CLOS residues were detectable above CCα in milk samples at levels of 432, 172, 156 and 41 μg kg^{-1} for 1, 2, 4, and 30 days post-treatment.

4. Conclusions

A multi-class UHPLC–ESI-MS/MS assay has been developed for the quantitative (two transitions for each analyte) and qualitative analysis (identification points, ion ratios, S/N ratio) of 38 anthelmintic veterinary drug residues in milk according to 2002/657/EC. The QuEChERS UHPLC–MS/MS assay has many benefits compared with traditional HPLC and LC–MS/MS methods without polarity switching such as; high sample throughput due to reduced run time and only one injection required per sample, reduced labour, waste and solvent usage. Sample throughput is twice that of LC–MS/MS methods and solvent usage one-fifth of HPLC based methods. This paper highlights how quick, easy, cheap, effective and rugged the QuEChERS extraction method. The combination of removing water, proteins, fat and contaminants, and extracting the analytes in one step and the DSPE reduce the extraction time significantly while maintaining high recoveries. The suitability of the assay has been assessed through application to proficiency test and incurred milk samples. This assay is very sensitive with CCα values in the range of 0.14–1.9 μg kg^{-1} for unapproved substances, which include many flukicide and avermectin substances. The multi-residue UHPLC–MS/MS method described is the most sensitive method available to detect a wide range of anthelmintic residues in milk.

Acknowledgements

This research was funded by the Irish Department of Agriculture, Fisheries and Food under the Food Institutional Research Measure as part of the National Development Plan (Projects 06RDTMFRC434 and 06RDTAAFRC479) and by EU Sixth Framework programme on Food Quality and Safety, ProSafeBeef project FOOD-CT-2006-36241. The Federal Office of Consumer Protection and Food Safety (BVL) (European Community Reference Laboratory) is acknowledged for support through organisation of proficiency test studies. James Morphet (Waters, Manchester) is acknowledged for technical support in setting up of UHPLC–MS/MS conditions. Patrick Whelan is acknowledged for supplying incurred milk samples.

References

[1] W.D.Z. Lopes, T.R. dos Santos, F.D. Borges, C.A.M. Sakamoto, V.E. Soares, G.H.N. Costa, G. Camargo, M.E. Pulga, C. Bhushan, A.J. da Costa, Vet. Parasitol. 166 (2009) 98.
[2] T.S. Waghorn, D.M. Leathwick, A.P. Rhodes, R. Jackson, W.E. Pomroy, D.M. West, J.R. Moffat, N. Z. Vet. J. 54 (2006) 278.
[3] R.E. Baynes, M. Payne, T. Martin-Jimenez, A.R. Abdullah, K.L. Anderson, A.I. Webb, A. Craigmill, J.E. Riviere, J. Am. Vet. Med. Assoc. 217 (2000) 668.
[4] European Agency for the Evaluation of Medicinal Products, EMEA/MRL/452/98-FINAL, Nitroxinil Summary Report, 1998.
[5] M. Danaher, L.C. Howells, S.R.H. Crooks, V. Cerkvenik-Flajs, M. O'Keeffe, J. Chromatogr. B 844 (2006) 175.
[6] M. Danaher, H. De Ruyck, S.R.H. Crooks, G. Dowling, M. O'Keeffe, J. Chromatogr. B 845 (2007) 1.
[7] D.L. Brandon, A.H. Bates, R.G. Binder, W.C. Montague, L.C. Whitehand, S.A. Barker, J. Agric. Food Chem. 50 (2002) 5791.
[8] S.R.H. Crooks, B. McCarney, I.M. Traynor, C.S. Thompson, S. Floyd, C.T. Elliott, Anal. Chim. Acta 483 (2003) 181.
[9] J. Keegan, M. Whelan, M. Danaher, S. Crooks, R. Sayers, A. Anastasio, C. Elliott, D. Brandon, A. Furey, R. O'Kennedy, Anal. Chim. Acta 654 (2009) 111.
[10] J.V. Samsonova, G.A. Baxter, S.R.H. Crooks, C.T. Elliott, J. AOAC Int. 85 (2002) 879.
[11] D. Fletouris, N. Botsoglou, I. Psomas, A. Mantis, J. AOAC Int. 79 (1996) 1281.
[12] K. Takeba, K. Fujinuma, M. Sakamoto, T. Miyazaki, H. Oka, Y. Itoh, H. Nakazawa, J. Chromatogr. A 882 (2000) 99.
[13] H.W. Sun, F.C. Wang, L.F. Ai, J. Chromatogr. Sci. 46 (2008) 351.
[14] H.W. Sun, F.C. Wang, L.F. Ai, J. Chromatogr. A 1175 (2007) 227.
[15] D.A. Durden, J. Wotske, J. AOAC Int. 92 (2009) 580.
[16] D.A. Durden, J. Chromatogr. B 850 (2007) 134.
[17] R. Sheridan, L. Desjardins, J. AOAC Int. 89 (2006) 1088.
[18] P. Jedziniak, T. Szprengier-Juszkiewicz, M. Olejnik, J. Chromatogr. A 1216 (2009) 8165.
[19] B.J.A. Berendsen, P.P.J. Mulder, H.A. van Rhijn, Anal. Chim. Acta 585 (2007) 126.
[20] K. Takeba, T. Itoh, M. Matsumoto, H. Nakazawa, S. Tanabe, J. AOAC Int. 79 (1996) 848.
[21] G. Dowling, H. Cantwell, M. O'Keeffe, M.R. Smyth, Anal. Chim. Acta 529 (2005) 285.
[22] D. Vuckovic, E. Cudjoe, D. Hein, J. Pawliszyn, Anal. Chem. 80 (2008) 6870.
[23] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal. Chim. Acta 637 (2009) 196.
[24] M.M. Aguilera-Luiz, J.L.M. Vidal, R. Romero-Gonzlez, A.G. Frenich, J. Chromatogr. A 1205 (2008) 10.
[25] R.J.B. Peters, Y.J.C. Bolck, P. Rutgers, A.A.M. Stolker, M.W.F. Nielen, J. Chromatogr. A 1216 (2009) 8206.
[26] A.G. Frenich, J.L.M. Vidal, R. Romero-González, M.d.M. Aguilera-Luiz, Food Chem. 117 (2009) 705.
[27] R. Ventura, R. Ramrez, N. Monfort, J. Segura, J. Pharm. Biomed. Anal. 50 (2009) 886.
[28] D.A. Bohm, C.S. Stachel, P. Gowik, J. Chromatogr. A 1216 (2009) 8217.
[29] T. Kovalczuk, O. Lacina, M. Jech, J. Poustka, J. Hajslova, Food Addit. Contam. 25 (4) (2008) 444.
[30] C.C. Leandro, P. Hancock, R.J. Fussell, B.J. Keely, J. Chromatogr. A 1144 (2007) 161.
[31] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC).
[32] E.H. McIlhenny, K.E. Pipkin, L.J. Standish, H.A. Wechkin, R. Strassman, S.A. Barker, J. Chromatogr. A 1216 (2009) 8960.
[33] I. Tarcomnicu, M.C. Gheorghe, L. Silvestro, S.R. Savu, I. Boaru, A. Tudoroniu, J. Chromatogr. B 877 (2009) 3159.
[34] J.T. Goras, A.R. Gauthier, J. AOAC 68 (1985) 598.
[35] Anon. Environmental Impact Analysis Report, Pfizer Inc., 235 East 42nd Street, New York 10017, 1982, p. 149.